PERGAMON

Vision Research 41 (2001) 1755–1763

Vision Research

www.elsevier.com/locate/visres

Ionochromic properties of long-wave-sensitive cones in the goldfish retina: an electrophysiological and microspectrophotometric study

Pavel P. Zak [a], Mikhail A. Ostrovsky [a], James K. Bowmaker [b,*]

[a] *Laboratory of Sensory Reception, Institute of Biochemical Physics, Kosygin Str 4, 117334 Moscow, Russia*
[b] *Department of Visual Science, Institute of Ophthalmology, University College London, London EC1V 9EL, UK*

Received 31 August 2000; received in revised form 16 January 2001

Abstract

Long-wave-sensitive (LWS) cone visual pigments are sensitive to the concentration of chloride ions and show a spectral shift to shorter wavelengths when exposed to low chloride levels. We have used the aspartate-isolated mass receptor potential of the electroretinogram (ERG) to establish whether the spectrally shifted cone pigment is functional. In the goldfish, *Carassius auratus*, the λ_{max} of the LWS porphyropsin is displaced from about 622 nm to around 606 nm when chloride is replaced by gluconate. The electrical response of the LWS cones (but not MWS cones and rods) is selectively and reversibly abolished by the removal of chloride ions. The effect is concentration dependent and a decrease to half the normal chloride ion concentration is sufficient to cause a decrease in the response.

Keywords: L cone; Mass receptor potential; Visual pigment anion sensitivity; Classification: Teleostei, Cyprinidae, *Carassius auratus*

1. Introduction

A notable feature of vertebrate long-wave-sensitive (LWS) visual pigments, those with absorbance maxima (λ_{max}) longer than about 520 nm, is that their spectral locations are determined not only by the specific amino acid sequences of their opsins, but also by their anionic environment. Crescitelli (1977) reported that long-wave visual pigment of the gecko (*Gecko gecko*) can be displaced to shorter wavelengths, from 521 nm to about 505 nm, by lowering the chloride ion concentration. A similar 'blue'-shift was also reported for the chicken LWS cone pigment (Fager & Fager, 1979; Knowles, 1976, 1980; Slobodyanskaya, Abrashin, & Ostrovsky, 1980) and has since been found in the LWS cone pigments of all other vertebrate classes: teleosts, amphibians and mammals (Kleinshmidt & Hárosi, 1992; Novitsky, Zak, & Ostrovsky, 1989; Wang, Asenjo, & Oprian, 1993).

Using site-directed mutagenesis to substitute 18 different positively-charged amino acids in the opsins of the human LWS/MWS cone pigments, Wang et al. (1993) have identified two residues, histidine at site 197 and lysine at site 200 (primate LWS/MWS cone opsin numbering), as candidates for the chloride-binding site. These sites are in the extracellular loop of opsin that connects helices IV and V and close to the cysteine 203–cysteine 126 disulphide bond linking to the luminal end of helix III. The binding site is therefore in the vicinity of the Schiff's base counter ion, glutamate 129. His197 appears to be the dominant binding site, but Lys200 also participates in forming the chloride-binding pocket (Wang et al.). The two sites are conserved in nearly all LWS cone pigments (including the gecko LWS 'rod' pigment), but are substituted by glutamate and glutamine, respectively in all rod opsins and shorter-wave cone opsins. An exception is the 'green'-sensitive cone pigment of the mouse that, although being a member of the LWS family of opsins, has λ_{max} at about 508 nm, but does not possess a chloride binding pocket.

Although the hypsochromically shifted, chloride-free pigment is photosensitive and appears to 'bleach' in a manner similar to normal LWS cone pigments (Slobodyanskaya et al., 1980), the question remains as to whether it is functional in terms of its ability to initiate

* Corresponding author. Tel.: +44-20-76086832; fax: +44-20-7608685.
E-mail address: j.bowmaker@ucl.ac.uk (J.K. Bowmaker).

0042-6989/01/$ - see front matter
PII: S0042-6989(01)00033-5

transduction and to generate an electrophysiological response. We have attempted to answer the question by investigating the electrical responses of photoreceptors in isolated goldfish retinae perfused with salines containing varying concentrations of chloride. Since the chloride-binding site is situated in a hydrophilic pocket of opsin on the extracellular side of the membrane, we have assumed that it is easily accessible and that chloride may be readily exchanged.

The goldfish (*Carassius auratus*) was selected as a model system since its retina contains four spectrally distinct classes of cone pigment as well as rods. The cone complement consists of large unequal double cones containing a P620 and P535 in the two members, large single cones also containing either the P620 or P535, and two additional classes of small single cone possessing a P450 and a P380 (Fig. 1). The rods contain a P525 (Bowmaker, Thorpe, & Douglas, 1991b; Hárosi & MacNichol, 1974). All the pigments are porphyropsins. We have taken advantage of the relatively large spectral difference between the λ_{max} of the LWS and MWS cone pigments (separated by 80–90 nm) and the aim of the study was to compare the behaviour of the ionochromic LWS cones with that of the non-ionochromic MWS cones.

Absorbance spectra of isolated single cells were recorded by microspectrophotometry, whereas the electrical activity of the photoreceptors was studied by recording the aspartate-isolated mass receptor potential of the electroretinogram (ERG).

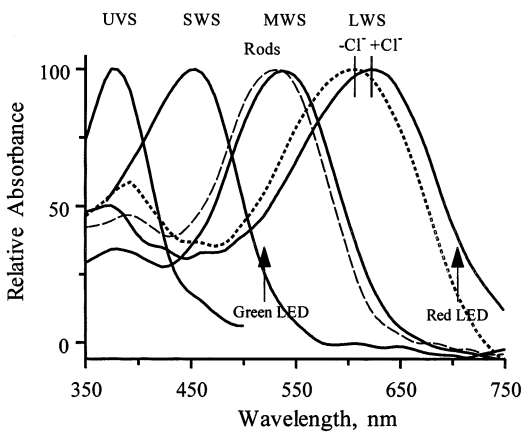

Fig. 1. Absorbance spectra of goldfish rods and cones, showing the relationship between the red and green stimuli to the relative sensitivities of the photoreceptors. The visual pigment spectra are normalised mean absorbance spectra obtained by microspectrophotometry. The LWS cones have λ_{max} at 622 nm in normal saline ($+Cl^-$), but 606 nm in chloride-free saline ($-Cl^-$) (for details see Table 2). Rods have λ_{max} close to 526 nm. The λ_{max} of MWS, SWS and UVS cones are about 535, 452 and 378 nm, respectively (data taken from Bowmaker et al., 1991b and Parry and Bowmaker, 2000). The arrows mark the maxima of the spectral distributions of the green (520 nm) and red (705 nm) stimuli.

2. Methods

2.1. Microspectrophotometry

Eyes were enucleated from fully dark-adapted goldfish, hemisected and the retinae removed under dim red light (Kodak Safelight No. 2). The retina was divided into two with one half maintained in normal saline and the other placed in the chloride-free solution. The salines were similar to those used in the electrophysiological experiments (see below). The retinae were incubated for at least 1 h in the chloride-free solution before measurements began. Absorbance spectra were recorded from the outer segments of both the LWS and MWS members of double cones and from rods. Spectra were recorded from 750 to 350 nm using a modified Liebman microspectrophotometer (Knowles & Dartnall, 1977; Liebman & Entine, 1964).

The methods of tissue preparation, spectral recording and data analysis were similar to those described previously (Bowmaker, Astell, Hunt, & Mollon, 1991a; Mollon, Bowmaker, & Jacobs, 1984). In summary, with the help of an infrared converter, the measuring beam (normally 2 μm square cross-section) was aligned to pass transversely through an outer segment while the reference beam passed through a clear space adjacent to the photoreceptor. Spectra were scanned from 750 to 350 nm in 2-nm steps and back from 351 to 749 nm at the interleaved wavelengths. To minimize the effects of bleaching, only one absorbance spectrum was obtained from a given outer segment, but two independent estimates of the baseline absorbance spectrum were obtained by arranging both beams to pass outside the cell.

A standardized computer programme was used to estimate the λ_{max} for each outer segment. First the two spectra from a cell were averaged and then the absorbance values at pairs of adjacent wavelengths were averaged to obtain a mean curve from the outward and return records. Each of the 20 absorbance values on the long wavelength limb of the curve (corresponding to a 40-nm segment and to absorbances in the range of approximately 45–90% of the maximum of the cell) was then referred to a standard template curve in order to obtain an estimate of λ_{max}. This operation amounts to finding the spectral location of a standard curve that gives the percent absorbance value under consideration. A second estimate of λ_{max} was obtained from the top of the absorbance curve by fitting each of 50 consecutive absorbance points, centred on the highest point, to the template curve and averaging the resulting estimates. The template curve used in the analysis was the Schwanzara standard curve for porphyropsin (Schwanzara, 1967) placed with its λ_{max} at 523 nm and expressed on an abscissal scale of log frequency, since absorbance curves of visual pigments have almost the same shape when expressed on such an abscissa (Bowmaker et al., 1991a; Mansfield, 1985).

Table 1
Effectiveness of red and green stimuli

Log I	Number of isomerizations/100 μm²/flash					
	Red flash			Green flash		
	LWS	MWS	SWS	LWS	MWS	SWS
0	7	–	–	1.5	6	2.3
1	70	–	–	15	60	23
2	700	–	–	150	600	230
3	7000	–	–	1500	6000	2300

Only records that passed rigid selection criteria were used for detailed analysis, the criteria for LWS and MWS cones being: (i) a transverse density at the λ_{max} greater than 0.01; (ii) a standard deviation from the right-hand limb estimate of λ_{max} of less than 5 nm; and (iii) the difference between the two estimates of λ_{max} less than 5 nm. The absorbance spectra from all cells of a given class that passed the criteria were averaged together to produce the mean spectra shown in Fig. 1, and it was from these averaged data that the λ_{max} values in Table 2 were obtained.

2.2. Electrophysiology

2.2.1. Preparation and procedure

Experiments were performed on isolated goldfish retinae, prepared as for microspectrophotometry. In order to improve the accessibility of the photoreceptors to the perfusate, excess vitreous was removed by gently brushing the retinal surface. Such retina preparations, when maintained in the cyprinid saline in the dark at 4–5°C, were viable for up to 24 h.

Two procedures for varying the chloride environment of the retina were employed. First, two retinae from the same fish or two halves of one retina were incubated for several hours in solutions of different chloride ion concentration. After incubation, the rod and cone responses were recorded from the retina, perfused with the incubation solution. Secondly, isolated retinae were perfused for relatively short periods of time by solutions of different chloride ion concentrations. In one set of experiments chloride was replaced by sulphate and in another by gluconate ($C_6H_{11}O_7^-$).

The following solutions, buffered to pH 7.45–7.50, were used:

1. normal chloride: NaCl 115 mM, Na-aspartate 20 mM, $NaHCO_3$ 10 mM, KCl 1.9 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 2.5 mM, dextrose 22 mM.
2. chloride-free, gluconate: Na-gluconate 115 mM, Na-aspartate 20 mM, $NaHCO_3$ 10 mM, K-gluconate 1.9 mM, Ca-gluconate 1.8 mM, $MgSO_4$ 2.5 mM, dextrose 22 mM.
3. chloride-free, sulphate: Na_2SO_4 57.5 mM, Na-aspartate 20 mM, $NaHCO_3$ 10 mM, K_2SO_4 0.95 mM, $CaSO_4$ 1.8 mM, $MgSO_4$ 2.5 mM, dextrose 22 mM.

The solutions with intermediate chloride content were prepared by mixing the normal chloride and chloride-free solutions as required.

2.2.2. Recording

The retina was placed photoreceptor-surface upward onto saline-soaked filter paper and dark-adapted for a further 40 min. Recordings were carried out at room temperature (about 20°C). The aspartate-isolated mass receptor potential (20 mM sodium aspartate) was recorded using Ag–AgCl electrodes, AC coupled, the system having a frequency bandpass of 0.3–30 Hz. The response amplitude of the perfused retina to a control light stimulus was stable for at least 3 h. In experiments where tissue was incubated at 4–5°C for several hours in solutions of different chloride ion concentration, recordings were made at 20°C from retinae perfused with the incubation medium. Again, recordings were stable for several hours.

2.2.3. Stimulus conditions

Short (0.02–20 ms), alternate red and green light flashes were used for retinal stimulation, with an interflash interval of either 1.0, 2.5 or 4.5 s. Red flashes were generated by a combination of a red photodiode and a red, 660-nm cut-off filter. Green flashes were generated by a combination of a green photodiode with a blue–green filter transmitting wavelengths shorter than 560 nm. As a result, the green stimulus had a peak at about 520 nm with a spectral bandwidth from 460 to 560 nm, whereas the red flash had a peak at about 705 nm with a bandwidth from 660 to 780 nm. The red flash primarily stimulated only the LWS cones, whereas the green flash excited all classes of photoreceptor except UVS cones.

Rod responses were induced by dim, threshold flashes presented to fully dark-adapted retinae. Under these conditions, slow responses characteristic of rods were recorded only to the green flash, but not to the red (Fig. 2A) which lies outside of the rod spectral sensitivity.

In order to isolate pure cone activity, rod responses were suppressed with a steady white background (10–25 cd/m²). Under these conditions the maximum response amplitude, V_{max}, was reduced (Fig. 2B), as would be predicted in the goldfish retina (Malchow & Yazulla, 1986). The intensities of the flashes covered four log units and this was sufficient to record the full range of cone sensitivity from threshold to V_{max} (Malchow & Yazulla, 1986). The ratio of intensities of the red and green stimuli was adjusted so that the amplitudes of the responses to the flashes were approximately equal. Typical records, made at all four log unit intensi-

ties, and 'response versus intensity' curves for the green and red flashes are shown in Fig. 2C. The effectiveness, in terms of the number of visual pigment isomerizations, of the intensities of the light stimuli in relation to the spectral sensitivities of the different types of cones is shown in Table 1. Since the green flash stimulated both the LWS and MWS cones, it was necessary to determine the contribution of the LWS cone response to the mass response. This was achieved by monitoring the effect of the responses to the red and green stimuli after selectively adapting the retina with either red (> 660 nm) or green (< 550 nm) background lights.

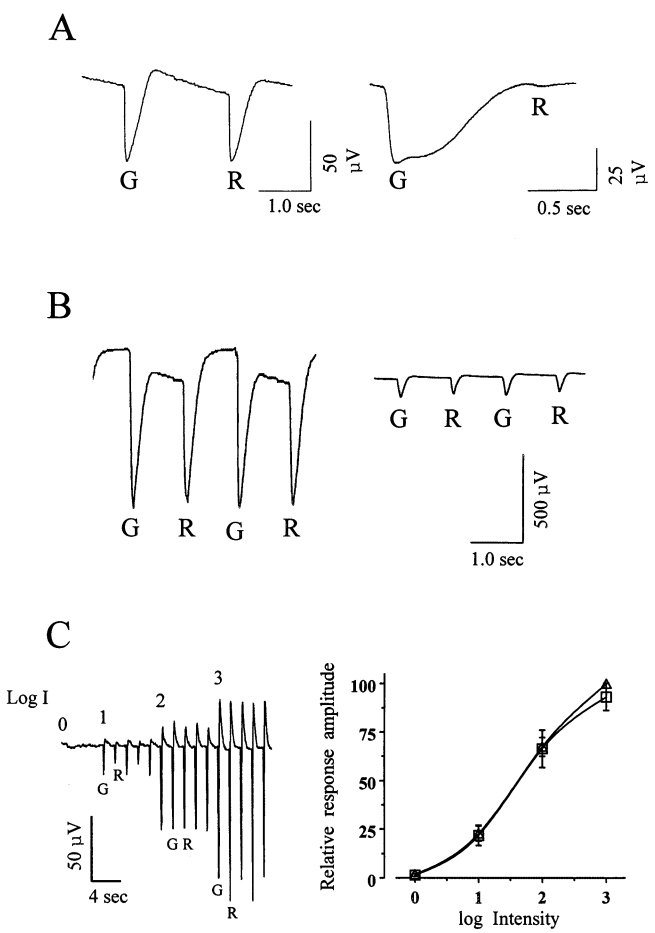

Fig. 2. Aspartate-isolated mass receptor potentials generated with alternate red (R) and green (G) flashes at different stimulus and background intensities. (A) Typical records generated from cones (left trace) and rods (right trace). Cone responses were recorded with a white background (25 cd/m^2) and high stimulus intensities (3 log units), whereas rod responses were obtained from fully dark-adapted retinae and dim flashes (1 log unit). (B) Isolation of cone activity after addition of a white background (25 cd/m^2) to abolish rod activity. Left trace: responses to 3 log unit intensity flashes with no background. Right trace: after application of the white background. (C) 'Response versus intensity' function for four stimulus intensities incrementing in 1 log unit steps in the presence of a white background (25 cd/m^2). Left: typical responses to alternate red and green flashes at the four stimulus intensities. Right: the response versus intensity functions for green (triangle) and red (square) flashes (mean of six experiments).

Table 2
Effect of chloride ions on λ_{max} of visual pigments

	Rods	MWS cones	LWS cones
Normal saline[a]	525.6 ± 2.5 ($n = 10$)	535.0 ± 1.7 ($n = 10$)	621.8 ± 4.2 ($n = 8$)
Chloride-free saline[b]	527.4 ± 2.2 ($n = 9$)	535.3 ± 1.4 ($n = 12$)	606.3 ± 3.6 ($n = 8$)
Difference	-1.8	-0.3	$+15.5$

[a] Normal saline contained NaCl 135 mM, $NaHCO_3$ 10 mM, KCl 1.9 mM, $CaCl_2$ 1.8 mM, dextrose 22 mM.
[b] Chloride-free saline had all chloride replaced with gluconate.

3. Results

3.1. Microspectrophotometry

The λ_{max} of the LWS and MWS cone pigments and rod pigment in normal saline were typical for goldfish, with λ_{max} at about 622, 535 and 526 nm, respectively, (Fig. 1). In contrast, in the chloride-free solution, the λ_{max} of the LWS cone pigment was displaced to shorter wavelengths with a λ_{max} close to 606 nm (Fig. 1). No such displacement was observed in the MWS cone or rod pigments whose λ_{max} remained at about 535 and 527 nm respectively (Table 2).

3.2. Electrophysiology

3.2.1. LWS cone contribution to the mass response to the green stimulus

An estimation of the LWS cones contribution to the mass response was obtained firstly by increasing intensities of a red background (> 660 nm). This selectively suppressed LWS cone activity. With complete adaptation of the red response, the amplitude of the response to the green flash was reduced to about 70% (Fig. 3A), suggesting that about 30% of the mass response to the green flash was contributed by the LWS cones.

A second estimation of the LWS cone contribution was obtained by adaptation to a blue–green background (< 550 nm). At low background intensities the amplitude of the green response decreased more rapidly than the red response, since the blue–green background will be more effective in adapting the MWS and SWS cones than the LWS cones. With increasing intensities of the background, both the green and red responses decreased at a similar rate. This suggests that at high background intensities, the responses of the SWS and MWS cones are fully inhibited, and only the LWS cones respond to the red and green flashes with a response ratio of about 3:1 (Fig. 3B). This value corresponds well with the relative numbers of photons absorbed by the LWS cones from the red and green flashes (see Table 1) and supports the conclusion that the LWS cones contribute about 30% of the mass response to the green flash.

3.2.2. Removal of chloride ions

After long-term incubation for a number of hours in the chloride-free solution, the response to the red flash was selectively abolished, whereas the responses to the green flash decreased by about 30–40% (Fig. 4A and B). The rod response was not affected by the long incubation in chloride-free solution (Fig. 4C). Control retinae, maintained in normal saline, showed very little reduction in response amplitudes.

In short-term perfusion experiments, the transition effects of replacing normal saline with low- or chloride-free saline were investigated. Cone activity was monitored using 1 and 2 log unit flash intensities that lay in the initial and middle regions of the 'response vesus intensity' function (Fig. 2C). Fig. 4D shows that after 5 min of perfusion with chloride-free saline, the response to the red flash at 1 log unit was lost, but the green response was maintained. At 2 log unit flash intensity there was a significant fall of the red response (by about 80%), but the response to the green flash decreased by only about 30%.

The reduction or abolition of the response to the red flash by the removal of chloride ions is reversible. Fig. 5 shows the effect of switching normal saline to a low-chloride (12.5%) perfusate. Reduction of the red response reached a maximum in about 18–20 min and, after switching back to normal saline, restoration to the initial amplitude of response occurred in about 5–10 min. In the case of the chloride-free perfusion experiments, the time taken for the red response reduction to a steady state level was about 5–10 min.

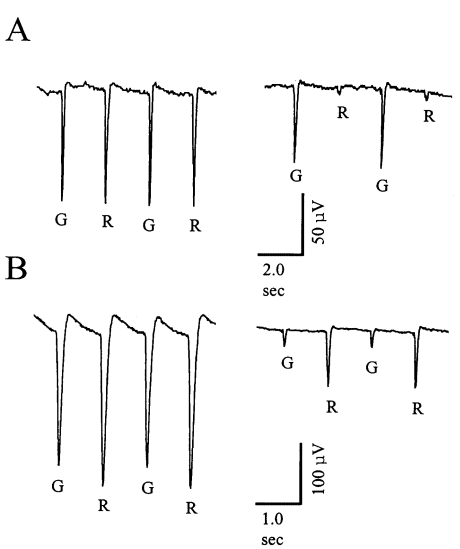

Fig. 3. The effect of selective spectral adaptation on the mass receptor potential. (A) Left trace: before addition of a red background (> 690 nm). Right trace: after adaptation with the red background. Stimulus intensity of 2 log units on a white background of 10 cd/m^2. (B) Left trace: before addition of a blue–green background (< 560 nm). Right trace: after adaptation with the blue–green background at an intensity where the responses to the red and green stimuli declined equally with increasing background intensity. Stimulus intensity of 2 log units on a white background of 10 cd/m^2.

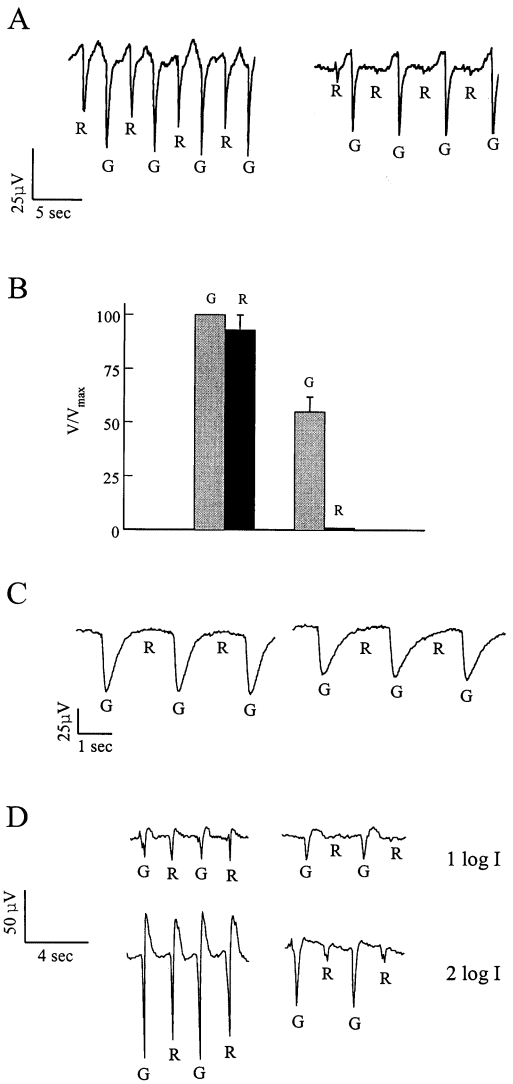

Fig. 4. Selective inhibition of LWS cone activity after application of chloride-free solution. (A) Left trace: responses in normal saline. Right trace: responses in chloride-free solution. Both recordings were made after incubation for 17 h. Stimulus intensity of 2 log units on a white background of 10 cd/m^2. (B) Mean relative amplitudes of cone responses in normal and chloride-free saline (data from six experiments). Left column pair: normal saline. Right column pair: after 4-h incubation in chloride-free solution. Stimulus intensity of 3 log units on a white background of 25 cd/m^2. (C) Rod responses. Left trace: normal saline. Right trace: after 4-h incubation in chloride-free saline. Stimulus intensity of 1 log unit on a fully dark-adapted retina. (D) Inhibition of LWS cone activity after short (5 min) perfusion with chloride-free saline. Upper traces: stimulus intensity of 1 log unit on a white background of 10 cd/m^2. Lower traces: stimulus intensity of 2 log units on the white background. Left traces: normal saline. Right traces: after 5 min perfusion with chloride-free saline.

3.2.3. Dependence of cone responses on the chloride concentration

In order to establish the relationship between a reduction in chloride ion concentration and the decrease in response to the red flash, retinae were perfused with solutions of different low chloride ion concentrations. The flash intensity was 2 log units. Measurements were taken when the amplitude decrease reached a steady-state level (normally after about 15–20 min), after which the perfusate was switched to normal saline and the responses were restored to their original levels. Fig. 6A illustrates typical records and Fig. 6B shows the mean 'response versus concentration' curve. A decrease in chloride ion concentration to 50% of the normal level was effective in producing a detectable reduction of the red response.

4. Discussion

4.1. Spectral shift and visual pigment activation

It is well established that removal of chloride ions from the medium causes a hypsochromic spectral shift in LWS visual pigments, but that the spectral locations of MWS cones and rods are insensitive to the anionic environment (Kleinshmidt & Hárosi, 1992). The shift of about 15 nm to shorter wavelengths, shown here for the LWS cones of *Carassius auratus*, when chloride ions are replaced by gluconate (Table 2), is almost identical to that reported for the LWS porphyropsins in *Helostoma temminckii* (kissing gourami) and *Ambystoma tigrinum* (tiger salamander) under similar ionic conditions (Kleinshmidt & Hárosi, 1992).

In addition to the spectral shift, the results of the electrophysiological experiments give clear evidence that there is a selective loss in sensitivity of the LWS cones with the removal of chloride ions. However, at low light intensities, where responses from the dark-adapted retina to the flash stimuli are dominated by rods, removal of chloride has no effect on the amplitude of the response to either red or green flashes. This supports the hypothesis that the visual pigments of rods are insensitive to the chloride ion concentration. In contrast, at photopic levels, the red response is significantly reduced. At least two possible hypotheses can be offered to explain this ionic effect: either the LWS cone pigment, spectrally displaced to shorter wavelengths and therefore less sensitive to long wavelengths, remains functional, or the spectrally shifted pigment is non-functional (or significantly less effective).

Suppression of the LWS cone response by lowered chloride concentrations should lead to a loss of response to the red flash and a concomitant reduction in response to the green flash. In contrast, in the case of a functional spectral displacement of the LWS cones, there should be only a decrease in the red response, with a small increase or almost no change in the green response. If the spectrally displaced pigment still maintains LWS cone function, then the reduction of the

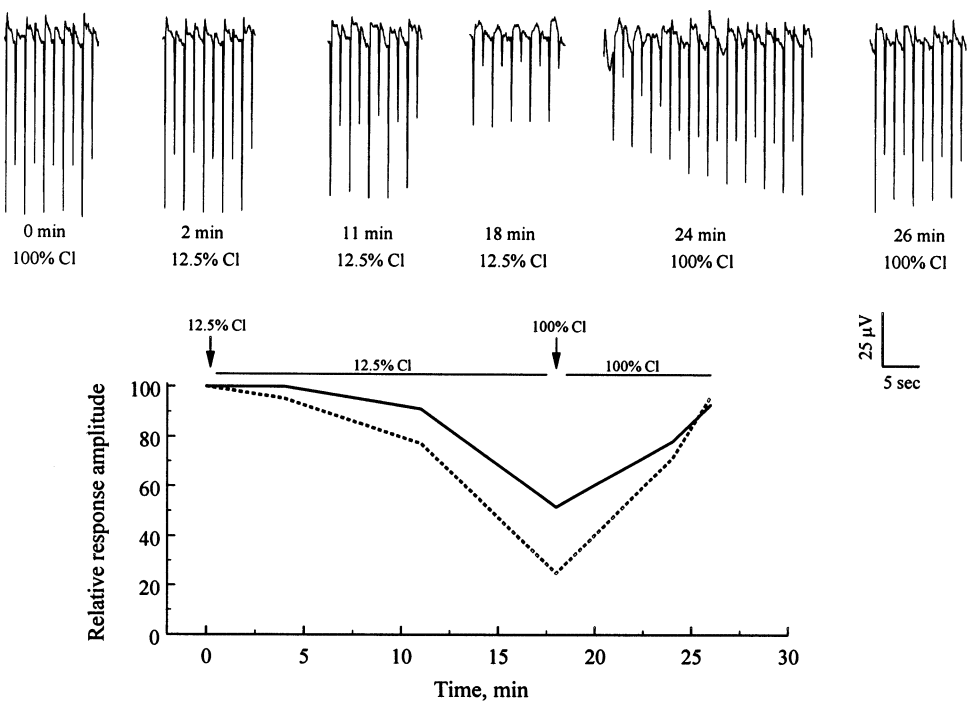

Fig. 5. Reversibility of the action of chloride-free saline on the cone responses under continuous perfusion. The chloride ion concentration was switched from 100 to 12.5% at time zero and returned to 100% after 18 min. Upper traces: responses to red and green flashes at times indicated. Lower graph: relative amplitudes of responses to red (dashed line) and green (solid line) flashes. Stimulus intensity of 2 log units with a white background of 15 cd/m^2.

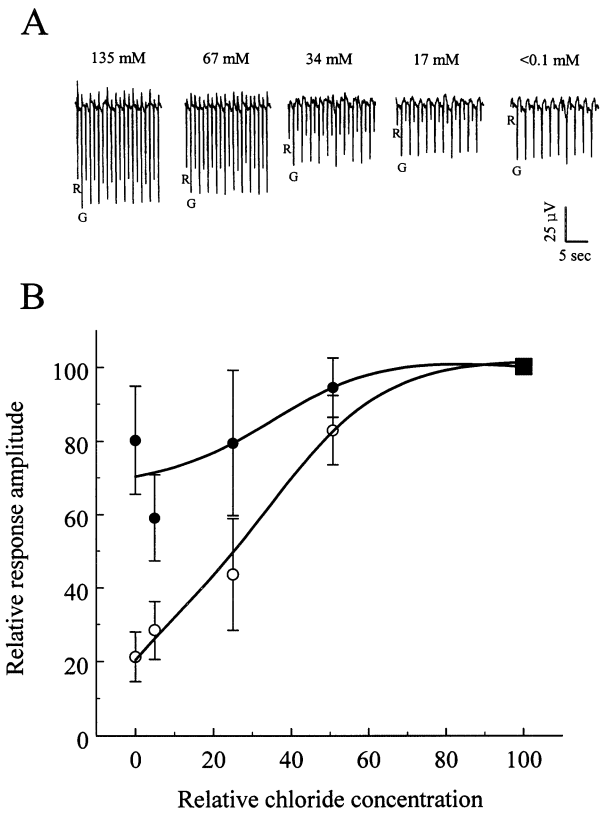

Fig. 6. Effect of different chloride ion concentrations on the cone responses. (A) Records of a typical experiment. The Cl^- ion concentrations are marked above each record. Stimulus intensity of 2 log units with a white background of 15 cd/m^2. (B) Relative amplitude of responses to red (filled circles) and green (open circles) flashes at different Cl^- ion concentrations. The data points are the means (with 95% confidence limits) from between seven and 17 experiments. The solid lines are least square fits to the data.

cone response to red flashes should be no more than 20%. This is simply because the 15-nm ionochromic shift will decrease the quantum catch of the visual pigment in the spectral band of the red stimulus by about 20%. However, if the LWS cones lose their ability to generate electrical responses in the absence of chloride ions, then the response to the red stimulus should be severely reduced or totally lost. In the case of the green response, if the LWS cone pigment is functionally displaced, the effect will be almost negligible. However, if the LWS cone activity is abolished, then the green response should fall by 30%, since this is the contribution of the LWS cones to the green response.

The data of Fig. 4 supports the first hypothesis, i.e. in the absence of chloride ions, the LWS cones cease to transduce (or the efficiency of transduction is severely reduced), but the MWS and SWS cones continue to function normally. If only the LWS cones cease to transduce in chloride-free saline, similar changes in the responses should be observed by applying a long-wave adapting light to the retina under normal saline conditions. This indeed was the case: the pattern of responses obtained with a long-wave adapting light, of sufficient intensity almost to abolish the response to the red flash, effectively mimicked the pattern of responses under reduced chloride conditions (cf. Fig. 3A and Fig. 4A). This reinforces the interpretation that the response to the red flash is almost solely from the LWS, whereas the response to the green flash, although dominated by the MWS cones, includes a significant contribution from LWS cones.

4.2. The specificity of the chloride effect on LWS cones

Chloride reduction was achieved by replacing chloride ions with either sulphate or gluconate ($C_6H_{11}O_7^-$). Although small non-organic sulphate ions and large organic gluconate ions possess different physical and chemical properties, their effects as a chloride substitute were identical, strongly suggesting that the observed effects can be specifically explained by the removal of chloride. Clearly, chloride is important for several processes in photoreceptor cells (e.g. Barnes, 1994). Chloride electrical conductance participates in the generation of the relaxation phase of the photoreceptor potential (Bader et al., 1982). In addition, chloride depletion suppresses transmitter release from photoreceptor terminals (Thoreson & Miller, 1996), though this action is unable to abolish the photoreceptor potential (Capovilla, Cervetto, & Torre, 1980; Thoreson, Nitzan, & Miller, 2000). Cobalt ions are able to specifically inhibit both these processes (Byzov & Cervetto, 1977; Capovilla et al., 1980; Cervetto & Piccolino, 1974), but does not abolish the aspartate-isolated mass receptor potential (Kapusta & Zak, 1994). This implies that the selective abolition of the aspartate-isolated mass receptor potential of the LWS cones cannot be explained by a disturbance of chloride ion conductance and/or transmitter release, but is most likely due to the ionochromic properties of the LWS cone visual pigment.

A complication that may arise through the use of gluconate is that it is able to bind calcium ions (Skibsted & Kilde, 1972). However, a reduction of calcium ion concentration leads to an increase, not a decrease, in the amplitude of the mass receptor potential (e.g., Sytchev, Zak, & Ostrovsky, 1977; Bochkin, Zak, & Ostrovsky, 1981). In any case, since the action of the sulphate solution was identical to that of the gluconate solution, the observed effects must be related to chloride depletion and not to gluconate addition.

4.3. Mechanism of chloride activation

At this stage, it is not possible to establish either the specific mechanism involved in the spectral effects of chloride binding or the processes that inhibit the func-

tion of the cone pigment when chloride is removed. A chloride ion, when bound to His197 in the hydrophilic domain on the extracellular region of opsin, possibly affects the charge of the Schiff's base counter ion complex dominated by Glu129. This may increase the delocalization of the π electron shell of the chromophore and displace the λ_{max} of the pigment to longer wavelengths. Removal of chloride ions will not only alter the charge associated with the counter ion, but also cause a modification in the tertiary structure of opsin by altering the relationship of the transmembrane helices (most likely helices III and IV). Such structural modifications could result in the photo-activated, chloride-free Meta II form of the pigment (R*) being unable to activate transducin. Activation of transducin involves highly conserved regions of the second and third cytoplasmic loops of opsin that connect helices III and IV, and helices V and VI. Mutations in these regions in rod opsins, which nevertheless yield photosensitive pigments with normal absorbance spectra, lead to pigments that fail either to bind transducin or, if bound, to activate it (Franke, Konig, Sakmar, Khorana, & Hofmann, 1990). Perturbations in helices III and IV of LWS cone pigments, caused by removal of chloride ions from their binding pocket on the extracellular side of the membrane, could lead to conformational changes in the cytoplasmic loops that, as with the mutated rod opsins, fail to bind or activate transducin.

The clear reversibility of the action of chloride on the electrical response of LWS cones suggests that the conformational changes in opsin are not very dramatic. The rather rapid effects imply a high accessibility of the chloride-binding site to the extracellular ionic medium and, since a distinct alteration of cone activity was observed at less than half normal chloride concentration, that the binding site probably has a relatively low affinity. The results also demonstrate that the importance of the chloride-binding site of LWS visual pigment is not only to produce the spectral shift to longer wavelengths, but also to stabilise the molecule into an appropriate conformational state necessary to activate phototransduction.

Acknowledgements

P.P.Z. and M.A.O. were supported at the Institute of Ophthalmology by the Royal Society of London.

References

Bader, C. R., Bertrand, D., & Schwartz, E. A. (1982). Voltage-activated and calcium-activated currents studied in solitary rod inner segments from the salamander retina. *Journal of Physiology*, *331*, 243–284.

Barnes, S. (1994). After transduction: response shaping and control of transmission by ion channels of the photoreceptor inner segment. *Neuroscience*, *58*, 447–459.

Bochkin, L. M., Zak, P. P., & Ostrovsky, M. A. (1981). Relationship between calcium and cyclic nucleotides in the retinal rods. *Sechenov Physiological Journal of the USSR LXVII*, *8*, 1194–1200.

Bowmaker, J. K., Astell, S., Hunt, D. M., & Mollon, J. I. (1991a). Photosensitive and photostable pigments in the retinae of Old World monkeys. *Journal of Experimental Biology*, *156*, 1–19.

Bowmaker, J. K., Thorpe, A., & Douglas, R. H. (1991b). Ultraviolet-sensitive cones in the goldfish. *Vision Research*, *31*, 349–352.

Byzov, A. L., & Cervetto, L. (1977). Effect of applied currents on turtle cones in darkness and during the photoresponse. *Journal of Physiology*, *265*, 85–102.

Capovilla, M., Cervetto, L., & Torre, V. (1980). Effects of changing the external potassium and chloride concentrations on the photoresponses of *Bufo bufo* rods. *Journal of Physiology*, *307*, 529–551.

Cervetto, L., & Piccolino, M. (1974). Synaptic transmission between photoreceptors and horizontal cells in the turtle retina. *Science*, *183*, 417–419.

Crescitelli, F. (1977). Ionochromic behavior of gecko visual pigments. *Science*, *195*, 187–188.

Fager, L. Y., & Fager, R. S. (1979). Halide control of color of the chicken cone pigment iodopsin. *Experimental Eye Research*, *29*, 401–408.

Franke, R. R., Konig, B., Sakmar, T. P., Khorana, H. G., & Hofmann, K. P. (1990). Rhodopsin mutants that bind but fail to activate transducin. *Science*, *250*, 123–125.

Hárosi, F. I., & MacNichol, E. F. (1974). Visual pigments of goldfish cones. Spectral properties and dichroism. *Journal of General Physiology*, *63*, 279–304.

Kapusta, N. V., & Zak, P. P. (1994). Extracellular photoreceptor potential of the white rat. *Biophisics*, *39*(3), 433–440.

Kleinshmidt, J., & Hárosi, F. I. (1992). Anion sensitivity and spectral tuning of cone visual pigments in situ. *Proceedings of the National Academy of Sciences*, *89*, 9181–9185.

Knowles, A. (1976). The effect of chloride ions upon chicken visual pigments. *Biochemical and Biophysical Research Communications*, *73*, 56–62.

Knowles, A. (1980). The chloride effect in chicken red receptors. *Vision Research*, *20*, 475–483.

Knowles, A., & Dartnall, H. J. A. (1977). The Photobiology of Vision. In H. Davson, *The eye*, *2B* (pp. 1–689). New York: Academic Press.

Liebman, P. A., & Entine, G. (1964). Sensitive low-level microspectrophotometer: detection of photosensitive pigments of retinal cones. *Journal of the Optical Society of America*, *54*, 1451–1459.

Malchow, R. P., & Yazulla, S. (1986). Separation and light adaptation of rod and cone signals in the retina of the goldfish. *Vision Research*, *26*(10), 1655–1666.

Mansfield, R. J. W. (1985). Primate photopigments and cone mechanisms. In J. S. Levine, & A. Fein, *The visual system* (pp. 89–106). New York: Alan Liss.

Mollon, J. D., Bowmaker, J. K., & Jacobs, G. H. (1984). Variations of colour vision in a New World Primate can be explained by polymorphism of retinal photopigments. *Proceedings of the Royal Society of London B*, *222*, 373–399.

Novitsky, I. Y., Zak, P. P., & Ostrovsky, M. A. (1989). The effects of anions on absorption spectrum of the longwavelenght retinal-containing pigment iodopsin in native frog cones (a microspectrophotometric study). *Bioorganic Chemistry*, *15*, 1037–1043.

Parry, J. W. L., & Bowmaker, J. K. (2000). Visual pigment reconstitution in intact goldfish retina using synthetic retinaldehyde isomers. *Vision Research*, *40*, 2241–2247.

Skibsted, L. H., & Kilde, G. (1972). Dissociation constant of calcium gluconate. Calculations from hydrogen ion and calcium ion activities. *Dansk Tidsskrift for Farmaci*, *46*, 41–46.

Slobodyanskaya, E. M., Abrashin, E. V., & Ostrovsky, M. A. (1980). A study of ionochromic properties of chicken visual pigments. *Bioorganic Chemistry*, *6*, 223–229.

Schwanzara, S. A. (1967). The visual pigments of freshwater fishes. *Vision Research*, *7*, 121–148.

Sytchev, V. S., Zak, P. P., & Ostrovsky, M. A. (1977). Ion Ca^{++} influence on time scale of frog photoreceptor potential. *Sechenov Physiological Journal of the USSR*, *LXIII*(11), 1541–1544.

Thoreson, W. B., & Miller, R. F. (1996). Removal of extracellular chloride suppresses transmitter release from photoreceptor terminals in the mudpuppy retina. *Journal of General Physiology*, *107*, 631–642.

Thoreson, W. B., Nitzan, R., & Miller, R. F. (2000). Chloride efflux inhibits single calcium channel open probability in vertebrate photoreceptors: Chloride imaging and cell-attached patch-clamp recordings. *Visual Neuroscience*, *17*, 197–206.

Wang, Z., Asenjo, A. B., & Oprian, D. D. (1993). Identification of the Cl^{-}-binding site in the human red and green colour vision pigments. *Biochemistry*, 32, 2125–2130.